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Effect of Three Concentration Techniques on Viability of *Cryptosporidium parvum* Oocysts Recovered from Bovine Feces

Z. BUKHARI[1,2] AND H. V. SMITH[1]*

Scottish Parasite Diagnostic Laboratory, Stobhill NHS Trust, Springburn, Glasgow G21 3UW,[1] and Division of Environmental Health, University of Strathclyde, Glasgow G1 1XQ,[2] Scotland

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Bovine fecal samples (1 g) negative for *Cryptosporidium* sp. oocysts were seeded with 7×10^4 *Cryptosporidium parvum* oocysts and purified by either water-ether concentration, sucrose density flotation, or zinc sulfate flotation to evaluate oocyst recovery. The effect of these purification techniques on the viability of recovered oocysts was also evaluated. Significantly higher numbers of seeded oocysts were recovered by water-ether concentration (recovery rate, 46 to 75%) than by sucrose density (24 to 65%) or zinc sulfate (22 to 41%) flotation methods. In addition, water-ether concentration did not exert a significant effect on the viability of the population of oocysts recovered, whereas sucrose density flotation and zinc sulfate flotation selectively concentrated viable oocysts. The water-ether concentration procedure is recommended for use in epidemiological studies in which both oocyst enumeration and viability assessment are required.

Techniques for the concentration and purification of oocysts from fecal samples include formol-ether concentration (1); sucrose density (4), zinc sulfate (7), and saturated salt solution (13) flotation; and Percoll discontinuous density gradient centrifugation (11). Whilst some of these techniques have been developed for the production of highly purified oocyst suspensions for biochemical, immunological, and molecular studies, others, such as formol-ether concentration and sucrose density and saturated salt solution flotation, have found favor for diagnosis and/or epidemiological studies, for which simple and effective oocyst concentration techniques are required. When attempting to assess the veterinary and/or public health significance of excreted oocysts, it is important to be able to determine the viability of populations of excreted oocysts as this provides an indication of the infectivity potential of those populations. Such an analysis demands that the oocyst concentration technique(s) should reflect accurately the proportions of viable and nonviable oocysts in the excreted population of oocysts. Failure to produce such a representation (e.g., by enriching the recovered oocyst populations with either viable or nonviable oocysts) will result in the misinterpretation of the significance of the results. Furthermore, the identification and utilization of a fecal purification technique that enables recovery of oocysts without exerting a significant effect on the viability of the recovered oocysts are important for examining the role of specific and nonspecific defense mechanisms in oocyst viability. In this study, *Cryptosporidium parvum* oocysts of predetermined viability were seeded into preweighed bovine feces and purified by either water-ether concentration, sucrose density flotation, or zinc sulfate flotation in order to evaluate both oocyst recovery and the effect of these techniques on the viability of the recovered oocyst populations.

MATERIALS AND METHODS

Source of purified oocysts. The cervine-ovine isolate of *C. parvum* oocysts, originally isolated from the feces of red deer calves (*Cervus elaphus*) (2) and maintained by passage through lambs, was obtained from the Moredun Research Institute, Edinburgh, United Kingdom. Oocysts were isolated and purified from lamb feces according to the method described by Hill et al. (6) and stored in Hanks' balanced salt solution (HBSS), containing antibiotics, at 4°C until used.

Calf feces, in which *C. parvum* oocysts could not be detected following water-ether concentration and fluorescein isothiocyanate-labelled anti-*Cryptosporidium* sp. monoclonal antibody (FITC-MAb) detection, were divided into 1-g quantities and each resuspended in 5 ml of grade 1 water (deionized water which has undergone reverse osmosis; conductivity, 0.0 to 0.09 μS/cm; British standard no. 3978). Stock suspensions of oocysts (<2 months old) were enumerated both with an Improved Neubauer hemocytometer and FITC-MAb and diluted in grade 1 water to provide a concentration of 1.4×10^5 ml^{-1}. Each fecal suspension was seeded with 500 μl of the stock solution containing 7×10^4 *C. parvum* oocysts and mixed thoroughly. Each suspension was made up to 50 ml with grade 1 water, vortexed, and centrifuged (1,050 × *g*, 5 min). The pellets were resuspended, and the washing process was repeated a further two times. The final pellets were adjusted to 10 ml (or 2 ml for zinc sulfate flotation) in grade 1 water and subjected to either the sucrose density flotation or the water-ether concentration method.

Evaluation of fecal purification techniques. (i) Sucrose density flotation. Washed fecal samples (10 ml) were underlaid with an equal volume of cold sucrose (specific gravity, 1.18) and centrifuged (1,050 × *g*, 15 min). Following centrifugation, 10 ml of the fluid interface (containing the oocysts) was removed, washed three times in grade 1 water, and resuspended to a final volume of 5 ml.

(ii) Water-ether concentration. Briefly, 2 ml of diethyl ether was added to the washed fecal samples (10 ml) and the samples were vortexed for 20 s and then centrifuged (1,050 × *g*, 5 min). Both the fat layer and the supernatant were discarded, and the pellet was resuspended in 50 ml of grade 1 water and centrifuged (1,050 × *g*, 5 min). This washing procedure was repeated twice, and the pellet was resuspended in grade 1 water to a final volume of 5 ml.

(iii) Zinc sulfate flotation. Washed fecal samples (2 ml) were thoroughly mixed in 10 ml of zinc sulfate solution (specific gravity, 1.18) and centrifuged (1,050 × *g*, 2 min). Following centrifugation, 2 ml of fluid from the meniscus (containing the oocysts) was removed and washed three times in grade 1 water, and the pellet was resuspended to a final volume of 5 ml.

Enumeration of purified oocysts. A 100-μl aliquot of concentrate from each of the purification techniques was air dried and methanol fixed onto microscope slides and stained with a commercially available FITC-MAb which recognizes oocyst-specific surface-exposed epitopes (Shield Diagnostics Ltd., Dundee, United Kingdom) according to the manufacturer's instructions.

Assessment of viability of *C. parvum* oocysts. The viabilities of both the stock suspensions and the oocysts recovered following the concentration techniques were assessed by the fluorogenic vital-dye assay (3). Furthermore, for selected samples the viability assessment method of maximized in vitro excystation (10) was also performed.

Fluorogenic vital-dye assay. An aliquot (20%) of each purified fecal sample was acidified by incubation in acidified HBSS (pH 2.75, 1 h, 37°C). Each sample was washed three times with HBSS (pH 7.2) and concentrated (12,500 × *g*, 30 s) to 100 μl, and 10 μl of 2-mg ml^{-1} 4′6-diamidino-2-phenylindole (DAPI) and 10 μl of 1-mg ml^{-1} propidium iodide were added. Samples containing the dyes were

* Corresponding author. Phone: 44 (0) 141 201 3028. Fax: 44 (0) 141 558 5508.

TABLE 1. Enumeration and percent recovery of *C. parvum* oocysts following three oocyst concentration techniques

Oocyst concn technique	Range of:		Coefficient of variation (%)
	Oocysts enumerated per slide	% Recovery	
Water-ether concn[a]	649–1049	46.4–74.9	11.7
Sucrose density flotation[b]	336–907	24–64.8	26.0
Zinc sulfate flotation[c]	303–572	21.6–40.9	16.8

[a] $n = 26$.
[b] $n = 21$.
[c] $n = 12$.

incubated at 37°C for 2 h. Optimally diluted FITC-MAb was added after 90 min of incubation with the two dyes, and the samples were incubated (37°C) for a further 30 min. Following a 2-h incubation with the dyes, each sample was washed three times with HBSS (pH 7.2) and concentrated to 100 μl, and then 10-μl aliquots were placed on glass microscope slides, covered with coverslips, and examined by microscopy.

Maximized in vitro excystation. Acidified (see above) aliquots (20%) of purified fecal samples were incubated with 200 μl of 1% bile solution (prepared in 1% Hanks Minimal Essential Medium [HMEM]) and 50 μl of sodium bicarbonate (0.44% in grade 1 water) at 37°C. After 30 min of incubation an aliquot of the sample was removed to observe sporozoite release, and after 4 h of incubation at 37°C percent excystation was determined (10).

Microscopy. Microscopy was performed (magnifications, ×40 for the objective and ×12.5 for the eyepieces) with an Olympus BH2 fluorescence microscope, equipped with Nomarski DIC (differential interference contrast) optics. A blue filter (excitation, 480 nm; emission, 520 nm) was utilized for the detection of FITC-MAb-labelled oocysts, and viability was assessed by using a UV filter block for DAPI (excitation, 350 nm; emission, 450 nm) and a green filter block for propidium iodide (excitation, 535 nm; emission, >610 nm).

Statistical analysis. The Mann-Whitney U test, F test, and t-test were used to compare both the oocyst recovery efficiencies and the effects of the concentration techniques on the viability of the concentrated oocysts. Determination of the correlation coefficient (r) between results obtained by the vital-dye assay and in vitro excystation was also performed by using a Microsoft Excel statistical package.

RESULTS

Analysis of enumeration. The number of *C. parvum* oocysts enumerated per sample (100-μl aliquots) and the calculated values for oocyst recovery are summarized in Table 1. Statistical evaluation of oocyst recoveries by water-ether concentration, sucrose density flotation, and zinc sulfate flotation indicated that the water-ether concentration method not only yielded significantly higher ($P < 0.05$) oocyst recovery rates but also yielded the lowest coefficient of variation (Table 1). Comparison of the numbers of oocysts recovered by sucrose density flotation and zinc sulfate flotation indicated that significantly higher ($P < 0.05$) oocyst recovery rates were obtained by sucrose density flotation.

When individual recoveries following water-ether concentration ($n = 26$) and sucrose density flotation ($n = 21$) were compared, 65.4% of samples enumerated following water-ether concentration were found to be above the range of recoveries by sucrose density flotation. Furthermore, no overlap between the ranges of oocyst recoveries was observed when recovery by water-ether concentration ($n = 26$) and zinc sulfate flotation ($n = 12$) were compared (Table 1).

Analysis of recovery rates following sucrose density flotation ($n = 21$) and zinc sulfate flotation ($n = 12$) indicated that 62% of the samples enumerated following sucrose density flotation yielded higher oocyst numbers than did those enumerated following zinc sulfate flotation.

Table 2 summarizes the mean oocyst numbers per slide and the mean recovery efficiencies for each multiple trial for each concentration technique.

The viabilities of the stock solutions of oocysts used to seed the fecal samples are presented in Table 3. When viabilities determined by the fluorogenic vital-dye assay and by the maximized in vitro excystation assay were compared, a mean of 84.7% (±6.1%; $n = 7$) were found to be viable according to the fluorogenic vital-dye assay and a mean of 83% (±4.2%; $n = 7$) were found to be viable according to the maximized in vitro excystation assay. The correlation coefficient between the vital-dye assay and maximized in vitro excystation for these seven samples was 0.92.

Effect of water-ether concentration, sucrose density flotation, and zinc sulfate flotation upon oocyst viability. Water-ether concentration demonstrated no significant ($P > 0.05$) effect on oocyst viability (Table 3) in comparison with control values. However, assessment of oocyst viability following sucrose density and zinc sulfate flotation (Table 3) indicated a significant ($P < 0.05$) increase in the viable oocyst populations, thus indicating that these flotation techniques selectively concentrate viable oocysts.

DISCUSSION

Similar numbers of oocysts (7×10^4) were seeded into oocyst-negative bovine fecal samples in our comparison of efficiencies of recovery by water-ether concentration, sucrose density flotation, and zinc sulfate flotation. Whereas water-ether concentration yielded recovery rates ranging from 46.4 to 74.9%, significantly lower oocyst recovery rates were obtained with the sucrose density flotation (24 to 64.8%) and zinc sulfate flotation (21.6 to 40.9%) techniques. Weber et al. (13) reported that sucrose flotation and zinc sulfate flotation yielded lower recovery rates than did the Formol-ethyl acetate sedimentation technique. Furthermore, Mtambo et al. (9), when evaluating seeded cat fecal samples, reported a higher oocyst recovery rate with a modified Formol-ether sedimentation technique than with either sucrose density or zinc sulfate flotation techniques. In addition, Waldman et al. (11), using an ether–phosphate-buffered saline concentration procedure, re-

TABLE 2. Recovery of *C. parvum* oocysts from bovine feces

Oocyst concn technique	Mean no. of oocysts/slide ± SD (mean % recovery ± SD)		
	Trial 1	Trial 2	Trial 3
Water-ether concn	960.2 ± 54.7[a] (68.6 ± 3.9)	785.6 ± 81.2[b] (56.1 ± 5.8)	876.8 ± 71.6[c] (62.6 ± 5.1)
Sucrose density flotation	698.7 ± 102.7[a] (49.9 ± 7.4)	589.5 ± 204.4[b] (42.1 ± 14.6)	667.3 ± 82.5[d] (47.7 ± 5.9)
Zinc sulfate flotation			475.2 ± 79.8[e] (33.9 ± 5.7)

[a] $n = 7$.
[b] $n = 11$.
[c] $n = 8$.
[d] $n = 3$.
[e] $n = 12$.

TABLE 3. Effects of three concentration techniques on viability of *C. parvum* oocysts recovered from seeded bovine feces

Technique	Result (% viable oocysts ± SD)					
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
Water-ether concn						
Control	84.7 ± 6.1[a]	60.4 ± 1.8[b]	71.5 ± 7.3[c]	71.8 ± 12.2[d]		
Posttreatment	82.5 ± 6.2[a]	58.2 ± 2.9[b]	70.0 ± 5.6[e]	72.0 ± 10.4[f]		
Sucrose density flotation						
Control		60.4 ± 1.8[b]	71.5 ± 7.3[c]		56.7 ± 3.8[d]	31.7 ± 2.9[f]
Posttreatment		75.2 ± 6.9[b]	83.0 ± 2.6[f]		76.7 ± 5.9[d]	66.8 ± 2.2[g]
Zinc sulfate flotation						
Control			71.5 ± 7.3[c]			
Posttreatment			81.8 ± 6.4[c]			

[a] $n = 7$.
[b] $n = 11$.
[c] $n = 12$.
[d] $n = 5$.
[e] $n = 8$.
[f] $n = 3$.
[g] $n = 4$.

ported recovery rates of up to 75% from calf feces. They proposed that ether sedimentation was better than sucrose flotation, as ether extracted lipids from the samples, thus dispersing the oocysts into the aqueous phase. In our study, water-ether concentration yielded the highest recovery rates; however, it is important to recognize that rates of oocyst recovery from feces can vary. *Cryptosporidium* sp. oocyst recoveries may be influenced by numerous factors. Increased recovery rates with either increasing numbers of oocysts (5) or watery (in comparison with formed) stools (12) have been documented.

When the effects of the purification procedures on the viability of the recovered oocysts were compared, it was found that water-ether concentration did not exert a significant effect on the viability of the recovered oocysts, whereas sucrose density and zinc sulfate flotation selectively concentrated viable oocysts. In contrast, for water samples LeChevallier et al. (8) reported that Percoll-sucrose gradients preferentially concentrated empty oocysts. Despite this, our examination of fecal pellets following sucrose density flotation confirmed the presence of large proportions of nonviable oocysts (2a). In wet preparations, clumps consisting of variable numbers of oocysts were occasionally observed, and determination of their viability status indicated that most of these clumped oocysts were nonviable (2a). This suggested that the presence of large proportions of nonviable oocysts in the sucrose pellets could be due to the exposed surface(s) of nonviable oocyst walls being more adhesive than those of their viable counterparts. The adhesion of nonviable oocysts to fecal debris and the subsequent sedimentation of the latter, following centrifugation, could account for the accumulation of nonviable oocysts in the sucrose pellets.

Although data from this investigation indicate water-ether concentration as the most appropriate method for use in epidemiological studies, some limitations in utilizing this technique exist. Ether is classed as an extremely flammable reagent and requires storage in suitable flammable-liquid storage cabinets; therefore, some laboratories utilize ethyl acetate as an alternative. In our evaluation of ether and ethyl acetate (data not shown), the former appeared to be more effective at extracting fats from stool samples and yielded cleaner fecal pellets. This is particularly important for detecting *Cryptosporidium* oocysts, which because of their small size (4 to 6 μm) can be easily occluded by contaminating fecal debris. A marginal reduction in oocyst viability was also noted following the use of ethyl acetate, although this reduction in oocyst viability was not statistically significant. When the use of ether is contraindicated, ethyl acetate can be substituted. However, as ethyl acetate is also flammable, individuals handling this reagent should take precautions against static discharges similar to those for ether and process samples in flameproof fume cupboards.

Proportionately smaller volumes of feces can be analyzed by sedimentation techniques than by flotation methods. Despite these limitations, our data indicate that sucrose density and zinc sulfate flotation do not provide an accurate representation of the percent viability of the oocyst population in feces. Therefore, in epidemiological studies, in which both oocyst enumeration and viability assessment are requirements, the selection of an oocyst concentration technique requires careful consideration. From our data, the water-ether concentration procedure appears to be the most appropriate.

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